Toxoplasma gondii infection blocks the development of allergic airway inflammation in BALB/c mice

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Summary

There is a link between increased allergy and a reduction of some infections in western countries. Epidemiological data also show that respiratory allergy is less frequent in people exposed to orofaecal and foodborne microbes such as *Toxoplasma gondii*. Infection with *T. gondii* induces a strong cell-mediated immunity with a highly polarized T helper type 1 (Th1) response in early stages of infection. Using a well-known murine model of allergic lung inflammation, we sought to investigate whether *T. gondii* infection could modulate the susceptibility to develop respiratory allergies. Both acute and chronic infection with *T. gondii* before allergic sensitization resulted in a diminished allergic inflammation, as shown by a decrease in bronchoalveolar lavage (BAL) eosinophilia, mononuclear and eosinophil cell infiltration around airways and vessels and goblet cell hyperplasia. Low allergen-specific immunoglobulin (Ig)E and IgG1 and high levels of allergen-specific IgG2a serum antibodies were detected. A decreased interleukin (IL)-4 and IL-5 production by lymph node cells was observed, while no antigen-specific interferon-γ increase was detected. Higher levels of the regulatory cytokine IL-10 were found in BAL from infected mice. These results show that both acute and chronic parasite infection substantially blocked development of airway inflammation in adult BALB/c mice. Our results support the hypothesis that *T. gondii* infection contributes to protection against allergy in humans.

Keywords: allergy, infections, lung, *Toxoplasma gondii*

Introduction

Pathology changes observed in bronchial asthma include production of increased allergen-specific immunoglobulin E (IgE) serum levels, predominant eosinophilic airway inflammation, increased mucus secretion and development of *in vivo* hyperreactivity dependent upon increased production of T helper type 2 (Th2) cytokines [1]. Several studies indicate that the incidence of atopic disorders (e.g. allergic rhinoconjuntivitis and asthma) has increased over the last decades, particularly in western countries [2,3]. Reasons for the increased prevalence of allergy are still not well understood. The pathogenesis of asthma reflects the influences of multiple factors including genetic susceptibility, infections and environmental exposures. Certainly, these two last factors are mainly responsible for the observed increment [4]. Epidemiological data allow establishing a link between decreased childhood infections and increased allergy in western countries [5–7]. It has been proposed that the protective effect of microbial exposure might be mediated, at least in part, by microbe-induced Th1 cytokines such as interferon (IFN)-γ [8]. Nevertheless, the immunological bases are controversial and while changes in Th2/Th1 balance are probably very important, they may not be the whole story.

Interestingly, epidemiological studies also showed that respiratory allergy is less frequent in people exposed to orofaecal and foodborne microbes such as *Toxoplasma gondii* and hepatitis A virus but not to viruses transmitted through other routes – mumps, chickenpox, herpes simplex virus type I, rubella and cytomegalovirus [9]. *T. gondii* has unique features to be considered as an allergy-modulating pathogen: (i) it is an obligate intracellular protozoan distributed globally; (ii) infection with the parasite leads to the induction of a strong cell-mediated immunity characterized by a highly polarized Th1 response in early stages of infection which is maintained during chronic infection [10,11]; and (iii) such an immune response predominates

and addresses the immune response of co-infected co-administered soluble proteins or parasites [12,13].

Toxoplasma has two asexual stages. The rapid dividing tachyzoites, found in the first week after infection in spleen, Peyer's patches and mesenteric lymph nodes and also in non-lymphoid tissues such as brain and liver, define the acute phase of infection. The slowly dividing bradyzoites, found inside tissue cysts mainly in brain, appear around the third week and mark the beginning of the chronic phase [14]. In mice, the parasite stimulates Toll-like receptor-11 and regulates the production of interleukin (IL)-12, necessary for the protective effect of IFN-γ [15]. High levels of IFN-γ and IL-12, as well as other proinflammatory cytokines, can be detected during the acute phase of infection. First natural killer cells and later, T cells, are the major source of IFN-γ [10,11]. Induction of the Th1 response occurs even in BALB/c mice that present a genetic predisposition for the development of a Th2 antigen-specific response [16]. Although the overwhelming levels of IFN-γ detected during the acute phase are no longer detected in the chronic phase, this cytokine is still the major mediator of host resistance during the chronic phase of infection [11,17,18]. When infected with the Beverley strain of *T. gondii*, a low virulent strain, BALB/c mice are resistant to developing toxoplasmic encephalitis [16]. Similarly, in human immunocompetent adults, toxoplasmosis is usually mild or clinically silent [19].

Despite epidemiological data and the features of the immune response associated with toxoplasmosis, until now the effect of *T. gondii* infection on allergy development has not been explored. In the present study, using a well-known mouse model of allergen sensitization and airway allergen challenges [20], we sensitized *T. gondii*-infected BALB/c mice with ovalbumin (OVA) to study whether the parasite can modulate susceptibility to developing respiratory allergies. Our results showed that *T. gondii* infection can block the development of allergic airway inflammation. This effect operates during both acute and chronic phases of infection.

Materials and methods

Animals

Female BALB/c mice 6–8 weeks old were obtained from the animal facilities of the ILEX-CONICET, IIHEMA Academia Nacional de Medicina (Buenos Aires) and maintained in our animal facilities for use throughout these experiments. The animals were housed according to the *NIH Guide for the Care and Use of Laboratory Animals*.

Infection, sensitization and exposure

The Beverley strain of *T. gondii* was used in this study. For infection, cysts were obtained from the brains of orally infected C3H/HeN mice and maintained by monthly passage. BALB/c mice were infected orally with 15 cysts. One week (acute phase) or 1 month (chronic phase) after infection, sensitization was achieved by two intraperitoneal (i.p.) injections of 0·2 ml phosphate-buffered saline (PBS) containing chicken OVA (grade III; Sigma-Aldrich, St Louis, MO, USA) (20 µg) and alum (2 mg) 1 week apart. One week later, mice were exposed to aerosols of allergen [3% (w/v))] OVA in PBS for 10 min on 3 consecutive days. Aerosol exposure was performed within individual compartments of a mouse pie chamber using a nebulizer (San-Up, Argentina, San Matin, Spain; OVA solution flux 0·33 ml/min in air flux of 6–8 l/min). Mice were analysed 48 h after the last exposure. Negative controls included *T. gondii*-infected and -non-infected mice both inoculated with alum alone and aerosolized with PBS.

Pathological analysis

Animals were killed with sodium pentobarbital. The chest wall was opened and the animals were exsanguinated by cardiac puncture. Serum was prepared and stored at − 20ºC. The trachea was cannulated after blood collection. Bronchoalveolar lavage (BAL) was performed four times with 1 ml of sterile PBS instilled and harvested gently. Lavage fluid was collected, centrifuged at 300 *g* for 10 min, and the pellet was resuspended in 0·5 ml PBS. Total cell yield was quantified and BAL differential cell counts were performed on cytocentrifuge slides prepared by centrifugation of samples at 80 *g* for 5 min (Cytospin 4; Shandon, Pittsburg, PA, USA). These slides were fixed and stained with a modified Wright–Giemsa stain (Tinción 15; Biopur SRL, Rosario, Argentina), and a total of 200 cells were counted for each sample by microscopy. Macrophages, lymphocytes, neutrophils and eosinophils were quantified. After lavage the lungs were instilled with 10% buffered formalin, removed and fixed in the same solution. Following paraffin embedding, sections for microscopy were stained with haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). An index of pathological changes in H&E slides was obtained by scoring the inflammatory infiltrates around the airways and vessels for greatest severity (0, normal; 1, ≤ 3 cells diameter thick; 2, 4–10 cells diameter thick; 3, ≥ 10 cells diameter thick) and overall extent (0, normal; 1, < 25% of sample; 2, 25–50%; 3, 51–75%; 4, > 75%). The index was calculated by multiplying severity by extent [21].

A histological goblet cell score was obtained in PAS-stained lung sections by examining 10–20 consecutive airways from all groups of mice at ×40 magnification and categorized according to the abundance of PAS-positive goblet cells (0, < 5% goblet cells; 1, 5–25%; 2, 25–50%; 3, 50–75%; 4, > 75%). The index was calculated by dividing the sum of the airway scores from each lung by the number of airways examined for the histological goblet cell score [22].

Assay of serum Ig

Anti-OVA-specific IgE, IgG1 and IgG2a were measured by enzyme-linked immunosorbent assay (ELISA). For IgE, 96-well microtitre plates (Nunc, Boston, MA, USA) were coated with 0·2 µg of monoclonal rat anti-mouse IgE (BD PharMingen, San Diego, CA, USA) diluted in 0·1 M carbonate buffer (pH 9·5). After incubation overnight at room temperature, plates were washed with PBS–0·05% Tween and blocked with PBS–bovine serum albumin (BSA) (5% w/v, pH 7·4) for 1 h, followed by addition of serum samples. After overnight incubation at room temperature and washing, biotinylated OVA (1 µg/ml) was added to the plates. Plates were incubated for 1 h at room temperature and washed. After another 1 h incubation with streptavidin–horseradish peroxidase (HRP) (Zymed, San Francisco, CA, USA; 1/4000), the reaction was developed with trimethylbenzidine substrate (TMB One-Step; Dako, Carpenteria, CA, USA). Plates were read in a Softmax plate reader (Molecular Devices, Menlo Park, CA, USA) at 655 nm [21]. For IgG1 and IgG2a, the plates were coated with OVA (10 µg/ml) and incubated overnight at 4oC. They were then washed with PBS–0·05% Tween and blocked with PBS–BSA (5% w/v, pH 7·4) for 1 h, followed by addition of serum samples. After incubation for 1 h at room temperature and washing, anti-mouse HRP–IgG1 or HRP–IgG2a (BD PharMingen) was added and incubated for 30 min at room temperature. For a standard curve, a monoclonal anti-OVA IgE (2C6) [21] and anti-OVA IgG1 (PLK3) were used. The ELISA for IgE and IgG1 can detect concentrations of 50 ng/ml of these antibodies when used as a standard control. For IgG2a, twofold serial dilutions of serum samples were added to the wells for determination of specific antibody titres. Titres were determined in duplicate for each serum sample and defined as the dilution that resulted in an optical density (OD) value greater than 0·1. Negative control serum samples included in the assay had OD values lower than 0·1.

In vitro stimulation and cytokine assays

Thoracic lymph nodes cells were harvested and 5×10^5 cells were cultured in 200 µl of medium RPMI-1640 supplemented with 20% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA), 1% antibiotics and 5×10^{-5} M 2-mercaptoethanol alone or in the presence of OVA (200 µg/ml) or concanavalin A (ConA) (5 µg/ml). For detection of IFN-γ secretion against *T. gondii* infection, 1 week or 30 days post infection (before OVA sensitization) 5×10^5 spleen cells were harvested and cultured for 48 h in medium RPMI-1640 supplemented with 20% FBS (Gibco), 1% antibiotics and 5×10^{-5} M 2-mercaptoethanol alone in the presence of soluble tachyzoite antigens [TgAg (25 µg/ml) or ConA (5 µg/ml)].

Cytokine production was measured in supernatants at 48 h (IFN-γ) or at 72 h (IL-4, IL-5 and IL-10) by capture ELISA commercial kits (Pharmingen, BD Biosciences). ConA-stimulated supernatants were harvested at 48h. Detection limit for all cytokines was 15 pg/ml.

Aspartate aminotransferase measurement

At the time of analysis normal and *T. gondii*-infected mice (days +32 and +55 after infection) were weighed, bled and levels of serum aspartate aminotransferase (AST) were determined by ultraviolet spectrophotometry using a commercial kit (Wiener Laboratory, Rosario, Argentina).

Statistical analysis

Each experimental group had at least four mice and each experiment was repeated at least four times. Data are presented as mean ± standard error of the mean (s.e.m.). Analyses of variance of differences among groups was performed with the Newman–Keuls test a posteriori. When variances differed significantly a non-parametric test (Kruskal–Wallis) was used with Dunns test a posteriori. Statistical significance was accepted when $P < 0.05$.

Results

Oral infection with cysts

BALB/c mice were infected experimentally by ingestion of cysts of the Beverly strain of *T. gondii* and 1 week later (acute phase of infection) or 30 days later (chronic phase of infection) they were sensitized and exposed to aerosols of OVA. *T. gondii* infection was confirmed by the presence of parasite-specific IgG antibodies (data not shown).

All infected mice were in good health, without signs of disease or weight loss. No difference in AST serum levels, measured at the time of the analysis (when the mice were killed) was observed between normal (33 ± 8 U/l, mean ± s.e.m.) and *T. gondii*-infected mice (32 days: 51 ± 9 U/l and 55 days: 37 ± 7 U/l), indicating that there were no hepatic or muscle lesions.

In order to evaluate the level of IFN-γ production in response to infection at the time of allergen sensitization, one group of mice was killed before OVA sensitization and cytokine production by spleen cells was analysed. Significant IFN-γ levels were detected in supernatants of splenocytes stimulated with TgAg during both acute (1902 ± 97 pg/ml, mean ± s.e.m.) and chronic stages of infection (347 ± 62 pg/ml). Similar to other studies [11], IFN-γ levels during the acute phase of infection resulted 5·5-fold higher compared with the chronic phase ($P < 0.05$). On the other hand, only non-stimulated splenocytes from acutely infected mice showed significant IFN-γ levels (75 ± 0·5 pg/ml). IFN-γ levels in negative controls (non-infected normal mice) were below the detection limit.
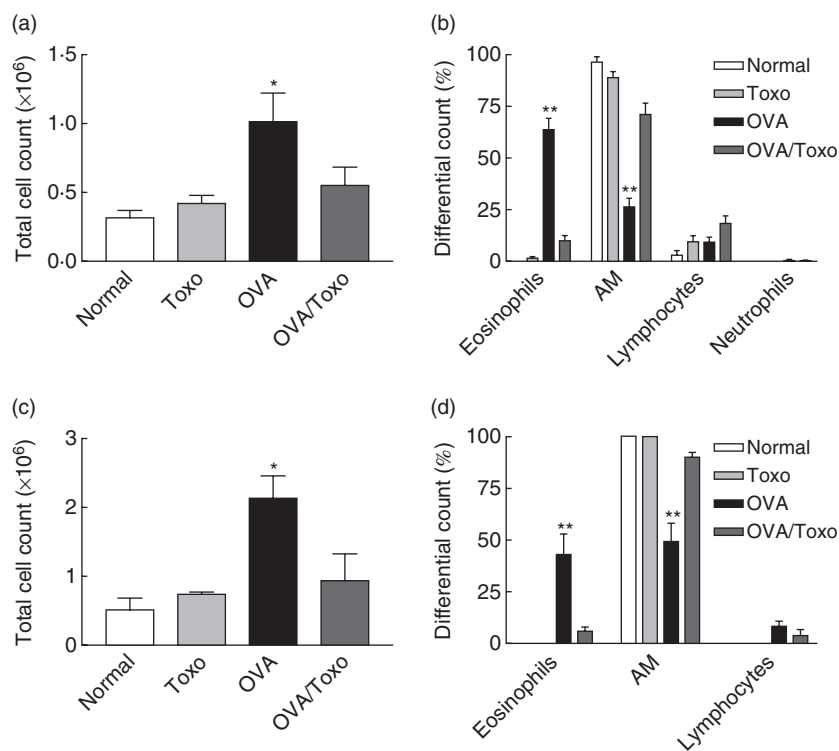
Fig. 1. Diminished eosinophilia in bronchoalveolar lavage (BAL). BAL was performed 48 h after the last exposure to ovalbumin. Total cell yield was quantified and BAL differential cell counts were performed on cytocentrifuge slides, fixed and stained with a modified Wright–Giemsa stain. (a, b) Results from mice sensitized during acute stage of infection; (c,d) the same for chronic treated mice. *$P < 0.05$ *versus* all other groups; **$P < 0.001$ *versus* all other groups.

Eosinophils in BAL

Increased total number of cells and accumulation of eosinophils in alveoli is a hallmark of bronchial asthma. Therefore, we analysed these two specific parameters in our experimental groups. As shown in Fig. 1a,c, allergic mice (OVA) showed an increased total number of BAL cells compared with normal and *T. gondii*-infected groups (TOXO), both acute (Fig. 1a) and chronic (Fig. 1c) ($P < 0.05$), which show a similar number of cells to normal mice. Allergic sensitization during both acute and chronic *T. gondii* infection (OVA/TOXO) showed a significant reduction in the total number of BAL cells ($P < 0.05$) compared with the OVA group, with results similar to the control group (Fig. 1a,c).

The differential count of BAL cells showed a significant increase in the percentage of eosinophils in the OVA group compared with the normal group ($P < 0.001$) (Fig. 1b,d). Infection with *T. gondii* before allergic sensitization resulted in a strong reduction of airway eosinophilia; indeed, eosinophils percentage and absolute numbers were diminished significantly. This effect was observed during both acute and chronic infection (Fig. 1b,d respectively). As shown in Fig. 1b,d, parasite infection does not affect the level of eosinophils in BAL. These results show that infection with *T. gondii* prior to allergic sensitization prevents the development of eosinophilia in BAL.

Lung pathology

The H&E and PAS lung-stained sections were analysed to evaluate whether suppressed BAL eosinophilia correlated

with reduced lung pathology. Figure 2 illustrates the lung pathology observed in PAS-stained sections from all experimental groups. Allergic mice (Fig. 2a) showed pathological changes of pulmonary allergic inflammation compared with normal mice (Fig. 2f). These changes include eosinophils and mononuclear cell infiltration around airway and vessels and mainly goblet cell hyperplasia. Mice infected with *T. gondii* before allergic sensitization showed a small inflammatory infiltrate with no goblet cell hyperplasia. This decrease in allergic lung inflammation was observed when mice were sensitized during both acute (Fig. 2b) and chronic phases of infection (Fig. 2c). Mice with *T. gondii* acute (Fig. 2d) or chronic infection (Fig. 2e) showed some cell infiltration not significantly different from normal mice. The results of semiquantitative scoring of histology support the qualitative changes described above (Table 1). As can be observed, mice sensitized during both acute and chronic stages presented inflammation and mucus indexes significantly lower than the allergic group.

Circulating allergen-specific IgE and IgG1

Atopy is characterized by high levels of allergen-specific IgE and Th2 IgG subsets. Thus, we analysed the *in vivo* effects of *T. gondii* infection on allergen-specific humoral immune responses in sera from the different experimental groups. Allergic senzitization resulted in high levels of IgE and IgG1 isotypes and low levels of IgG2a (Table 2). Infection with *T. gondii* before allergic sensitization decreased significantly the synthesis of Th2-directed IgE and IgG1 antibodies when compared with allergic mice, and this effect was observed

Table 1. Quantitative analysis of histopathological changes.

Group	Inflammation index[†]	Mucus index[‡]
Normal	0·4 ± 0·1	0 ± 0
TOXO acute	0·7 ± 0·2	0 ± 0
TOXO chronic	0·9 ± 0·5	0·2 ± 0·2
OVA	4·3 ± 0·5*	2·0 ± 0·2*
OVA/TOXO acute	2·2 ± 0·5**	0 ± 0
OVA/TOXO chronic	1·2 ± 0·2	0·6 ± 0·3

*$P < 0.001$ *versus* all other groups; **$P < 0.05$ *versus* normal. [†]An index of pathological changes in haematoxylin and eosin slides was obtained by scoring the inflammatory infiltrates around the airways and vessels for greatest severity (0, normal; 1, ≤ 3 cells diameter thick; 2, 4–10 cells diameter thick; 3, ≥ 10 cells diameter thick) and overall extent (0, normal, 1, < 25% of sample, 2, 25–50%, 3, 51–75%, 4, > 75%). The index was calculated by multiplying severity by extent. [‡]An histological goblet cell score was obtained in periodic acid-Schiff (PAS)-stained lung sections by examining 10–20 consecutive airways from all groups of mice at × 40 magnification and categorized according to the abundance of PAS-positive goblet (0, < 5% goblet cells; 1, 5–25%; 2, 25–50%; 3, 50–75%; 4, > 75%). The index was calculated by dividing the sum of the airway scores from each lung by the number of airways examined for the histological goblet cell score. OVA, ovalbumin; TOXO, *T. gondii*-infected.

during both acute and chronic phases. An enhancement of OVA-specific IgG2a, an IgG isotype driven by Th1 lymphocytes, was also detected in these two groups (Table 2).

Local Th2 cytokine production

In order to study the allergen-specific lymphocyte responses, we analysed by ELISA the concentration of cytokines in supernatants of thoracic lymph node cells after stimulation with OVA. As expected, allergic mice showed high production of both IL-4 and IL-5 compared with normal animals (Table 3). This predominant immune Th2 response was reversed when mice were infected previously with the parasite (Table 3). Indeed, infection with *T. gondii* before OVA sensitization resulted in a decrease in antigen-specific IL-4 and IL-5 production by thoracic lymph node cells both in acute and chronic infected mice. Regarding antigen-specific IFN-γ, no significant differences were detected between allergic mice and both of the previously infected groups. Nevertheless, a trend toward increased IFN-γ production was observed when mice were sensitized during acute infection
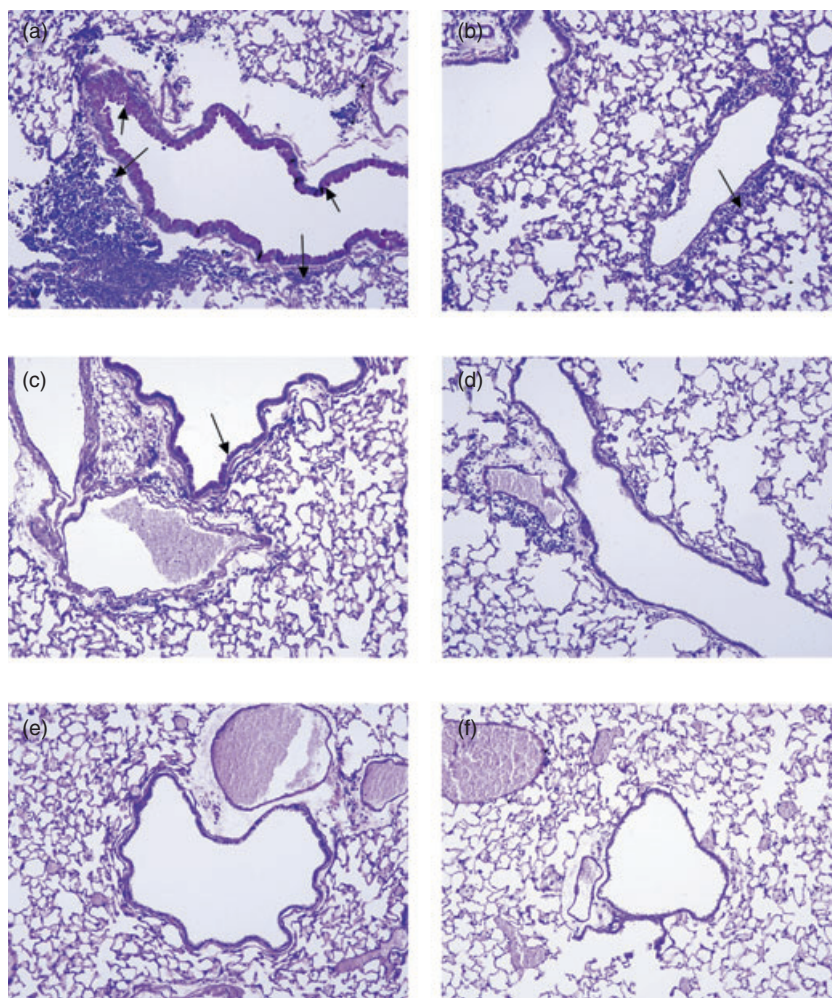

Fig. 2. Lung histopathology. After lavage lungs were instilled and fixed with 10% buffered formalin. Following paraffin embedding, sections for microscopy were stained with haematoxylin and eosin and periodic acid-Schiff. Histopathology showed a diminished infiltration around airways and vessels and no goblet cell hyperplasia in sensitized mice infected previously with *Toxoplasma gondii*. Arrows show prominent goblet cell hyperplasia and infiltration in allergic mice (a), compared with acute (b) and chronic (c) infected and sensitized animals. Negative control mice: (d) acute *T. gondii*-infected; (e) chronic *T. gondii* and (f) normal mice. Original magnification ×100.

Table 2. Effect of *Toxoplasma gondii* infection previous to allergic sensitization on immunoglobulin (Ig) production.

Group	IgE (ng/ml)	IgG1 (µg/ml)	IgG2a (titre) ($\times 10^4$)
Normal	< 50	$< 5.3 \times 10^{-4}$	n.d.
TOXO acute	< 50	$< 5.3 \times 10^{-4}$	< 0.01
TOXO chronic	< 50	$< 5.3 \times 10^{-4}$	< 0.01
OVA	420 ± 92*	155 ± 26*	0.8 ± 0.1*
OVA/TOXO acute	150 ± 90	79 ± 21	14 ± 3.4
OVA/TOXO chronic	214 ± 71	86 ± 32	5.8 ± 1.7

*$P < 0.05$ *versus* all other groups. Serum levels of ovalbumin-specific Ig were quantified in ovalbumin-sensitized mice (OVA), *T. gondii*-infected and OVA-sensitized (OVA/TOXO) (both groups aerosolized with OVA), *T. gondii*-infected (TOXO) or normal mice (both negative groups aerosolized with phosphate-buffered saline); n.d., not done.

(Table 3). A similar response pattern regarding IL-4 and IL-5 cytokines was obtained after stimulation with ConA. As expected, IFN-γ was detected in supernatants from ConA-stimulated cells from both sets of infected mice: OVA/TOXO and TOXO groups (acute and chronic) compared with allergic and normal mice. Nevertheless, a comparison between the levels of IFN-γ in OVA/TOXO and TOXO animals showed that they were significantly higher only in the OVA/TOXO acute infected group (Table 3).

Finally we measured IL-10, a cytokine that can be associated with both Th2 and regulatory responses. Indeed, it has been shown previously that allergic responses can be down-regulated by mediators such as IL-10. Herein, allergic mice showed high levels of production of this cytokine in thoracic lymph node cells supernatants after OVA stimulation, while sensitization during *T. gondii* infection induced a marked decrease in both acute and chronic groups (Table 3). The same tendency was detected when the cells were stimulated with ConA (Table 3). Interestingly, the results obtained when this cytokine was measured in BAL were quite different. Higher levels of IL-10 were detected in the OVA plus *T. gondii* groups (acute and chronic) compared with the allergic mice with values close to those found in non-sensitized infected animals (Fig. 3). Similar to the data obtained with

lymph node cells, IL-4 and IL-5 were decreased in the previously infected groups compared with allergic animals (Fig. 3). Regarding IFN-γ, no increase could be detected in *T. gondii*-infected plus OVA-sensitized groups (Fig. 3).

Discussion

Modulation of allergy by infections, such as mycobacteria [4], adenovirus [23] and helminths [24], or certain microbial products such as lipopolysaccharide (LPS) or CpG oligodeoxynucleotides [25–27] has been documented. Also, epidemiological data showed that respiratory allergy is less frequent in people exposed to orofaecal and foodborne microbes such as *T. gondii* and hepatitis A virus [9]. However, to our knowledge the effect of *T. gondii* infection on allergy development has not been studied. Using a well-known mouse model of allergen sensitization and airway allergen challenges we investigated the consequences of *T. gondii* infection on the development of pulmonary allergic inflammation. We demonstrated that BALB/c infection with the parasite suppresses antigen-induced airway inflammation as shown by the decrease in total eosinophils in BAL lavage, fewer peribronchial and perivascular infiltrates and mucin-producing cells. Interestingly, this protection was obtained during both acute and chronic parasite infection.

It has been proposed that less exposure to Th1-induced microbes could account for the increasing incidence of atopic disorders [8]. Resistance to *T. gondii* depends on IFN-γ production during both acute and chronic phases of infection. However, the peak of this Th1 cytokine occurs during the first week after infection (acute phase); later, approximately 30 days post-infection, lower levels are detected [11]. Indeed, we found that IFN-γ secretion by splenocytes from chronically infected animals was decreased compared with splenocytes from acutely infected mice. Even though lower IFN-γ levels are present during the chronic phase, *T. gondii* infection could still diminish the allergic lung inflammation.

Table 3. Effect of *Toxoplasma gondii* infection previous to allergic sensitization on allergen-specific and unspecific thoracic lymph node cells cytokine production *in vitro*.

Group	IL-4 (pg/ml)		IL-5 (pg/ml)		IFN-γ (pg/ml)		IL-10 (pg/ml)	
	OVA	ConA	OVA	ConA	OVA	ConA	OVA	ConA
Normal	< 15	30 ± 1	< 15	76 ± 14	< 15	247 ± 86	15 ± 7	285 ± 155
TOXO acute	< 15	196 ± 138	< 15	271 ± 88	< 15	1883 ± 200	29 ± 3	83 ± 52
TOXO chronic	< 15	123 ± 51	< 15	40 ± 4	< 15	1655 ± 64***	15 ± 1	72 ± 20
OVA	426 ± 146*	1096 ± 488	4220 ± 1155*	7105 ± 626*	28 ± 10	312 ± 38	479 ± 176*	903 ± 165
OVA/TOXO acute	23 ± 4	417 ± 143	62 ± 45	3042 ± 481	98 ± 38	4407 ± 1253**	48 ± 8	297 ± 97
OVA/TOXO chronic	27 ± 8	663 ± 271	427 ± 154	2122 ± 827	29 ± 25	2396 ± 498	35 ± 15	203 ± 101

*$P < 0.05$ *versus* all other groups; **$P < 0.05$ *versus* ovalbumin-sensitized (OVA), *T. gondii*-infected (TOXO) acute, TOXO chronic and normal mice; ***$P < 0.05$ *versus* normal mice. Cytokine production by thoracic lymph node cells cultured with OVA or concanavalin A (ConA) *in vitro* were measured in mice OVA-sensitized (OVA), *T. gondii*-infected and OVA-sensitized (OVA/TOXO) (both groups aerosolized with OVA), *T. gondii*-infected (TOXO) or normal mice (both negative groups aerosolized with phosphate-buffered saline). IL, interleukin; IFN, interferon.
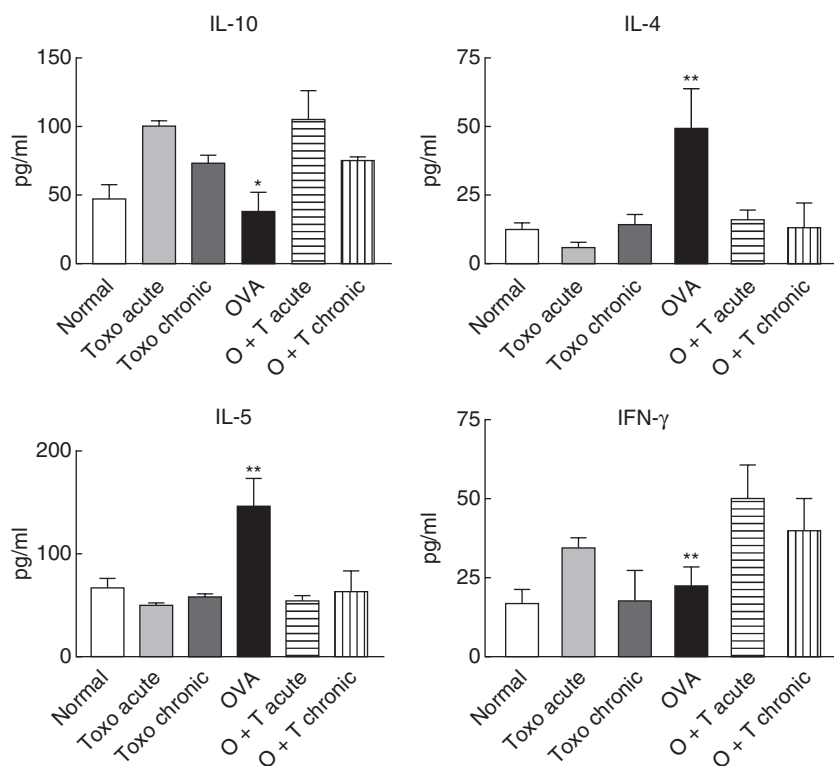
Fig. 3. *Toxoplasma gondii*-infected mice show increased bronchoalveolar lavage (BAL) interleukin (IL)-10 levels. BAL was performed 48 h after the last exposure to ovalbumin and cytokine levels were measured on BAL supernatant. *$P < 0.05$ *versus T. gondii*-infected and ovalbumin-sensitized (OVA/TOXO) acute and chronic, TOXO acute, TOXO chronic; **$P < 0.05$ *versus* all other groups.

Humoral immune response modulation of non-related antigens by *T. gondii* has been published previously [12]. It has also been shown that acute but not chronic infection with *T. gondii* protects BALB/c mice against *Leishmania major* by inducing an inhibitory effect on the development of Th2 lymphocyte-mediated responses [13]. In our model, OVA-specific humoral immune response in *T. gondii*-infected mice showed a shift in Ig isotypes from Th2 to Th1. A diminished level of OVA-specific IgE and IgG1 and an enhancement of IgG2a were detected when mice were sensitized during acute and chronic stages of infection. To our knowledge, this is the first work showing changes in IgE synthesis modulated by *T. gondii*. The ability of *T. gondii* infection to change the isotypic distribution of IgG antibodies against a non-related antigen biased in favour of IgG2a has been reported previously by Nguyen *et al.* [12]. Similar to our results, the Th1 deviation was observed during both phases of infection. In contrast, Santiago *et al.* [13] could not show enhancement of *Leishmania*-specific IgG2a when they analysed the *in vivo* effects of acute phase *T. gondii* infection on *L. major* humoral immune response.

The strongly diminished BAL IL-4 and IL-5 levels detected in OVA-sensitized acute and chronic infected mice compared with allergic animals suggest that the Th2 response to allergen exposure was reduced. These results correlated with those observed in supernatants of thoracic lymph node cells stimulated with OVA. The reduction in IL-5 by lymph node cells offers an explanation for the decreased eosinophilia in BAL, as this cytokine is involved in the production of eosinophils. These lower levels of BAL eosinophils may account, in turn, for the diminished IL-4 and IL-5 detected in BAL (both secreted in lung not only by Th2 cells but also by eosinophils) [28]. These data indicate that the *T. gondii* parasite could drive the immune response against OVA towards a Th1-associated profile. In fact, a trend to an increase in IFN-γ production from *T. gondii*-infected/OVA sensitized animals when stimulated *in vitro* with OVA, although not statistically significant, was observed only in the acute infected group. However, no changes in OVA associated-IFN-γ levels could be detected in thoracic lymph node cells compared with the chronic sensitized mice. No differences in IFN-γ levels could be detected in BAL from both infected groups. Similar to our results, protection against *L. major* by acute co-infection with *T. gondii* was accompanied with an enhancement of specific IFN-γ production by lymph node cells that were not significantly different from the *Leishmania*-infected mice [13]. Also, as already mentioned, adenovirus infection inhibited significantly the allergic inflammatory response in the lung tissue after antigen challenge [23]. Again, comparable to our model, the levels of IFN-γ in the BAL after antigen challenge were not increased in the adenovirus-infected group. Moreover, they were significantly lower than the allergic animals [23].

Taking all the data together, the clearest effect of *T. gondii* infection on this OVA-allergic model is to oppose the predominance of Th2-associated IL-4 and IL-5 cytokines as well as Th2-associated IgE and IgG1 isotypes against OVA. The

sensitization during the acute phase of *T. gondii* infection, when high levels of IFN-γ are detected, allows us to speculate that the protective effect is related to high concentrations of Th1 cytokines. These high levels of Th1 cytokines might, in part, inhibit the differentiation of Th precursors into Th2 lymphocytes and partially induce a shift towards a Th1 cellular response. Intervention experiments with neutralizing antibodies against IFN-γ would demonstrate the role of this cytokine in the protective effect, but this would abolish resistance of mice to *T. gondii* infection [11]. In the *Leishmania* model, the protective effect is not observed during the chronic phase of *T. gondii* infection [13]. In contrast, we still detect protection against allergy when we sensitize at this stage. What is the mechanism of action operating during chronic toxoplasmosis? We can hypothesize that the lower levels of IFN-γ present at this late stage in response to infection are sufficient to inhibit the differentiation of Th precursors into Th2 lymphocytes without driving a shift towards a Th1 lung cellular response. Nevertheless, other mechanisms could also be participating, even when sensitizing during acute toxoplasmosis.

The immunological bases of the protective effect of microbial exposure on allergy are still controversial. While changes in Th2/Th1 balance are probably very important, they may not be the only mechanisms involved. It has been shown that certain infections might protect against the development of allergy by inducing the production of regulatory cytokines such as IL-10 and transforming growth factor-β, which down-regulate both Th1 and Th2 responses [29–31]. Our data did not show higher levels of IL-10 in OVA or ConA-stimulated lymph node cells in infected mice. However, and interestingly, increased levels of this cytokine were detected in BAL from both acute and chronic infected mice. IL-10 was first described as a cytokine secreted by Th2 cells and accompanied the production of IL-4, IL-5 and IL-13 [32]. Control of Th2 cells is necessary to avoid the manifestation of pathologies and therefore production of IL-10, by limiting the Th2-cell response, reduces host damage [33]. It was shown that IL-10 prevents proinflammatory cytokine release from mast cells and suppresses IL-5 production by human resting Th0 and Th2 cells [34,35]. It has also been shown that IL-10 levels are correlated inversely with the incidence or severity of asthmatic and allergic disease [36,37]. Moreover, in animal models, transfer of the IL-10 gene to the airway [38] or adoptive transfer of IL-10 secreting CD4+ T cells [39] blocked airway inflammation. Takanaski *et al.* reported that IL-10 significantly inhibited survival of, and cytokine production from, human peripheral blood eosinophils induced by LPS [40]. It was observed subsequently that IL-10 induces apoptosis in human eosinophils by inhibiting the expression of CD40 [41]. Therefore, the diminished eosinophilia detected in this model might also be explained by the presence of IL-10 in BAL. Uncontrolled inflammatory Th1 cell responses, which eliminate intracellular pathogens, frequently cause immunopathology

and IL-10 controls this deleterious side effect [42]. Thus, IL-10 may be an important feedback regulator controlling various types of inflammatory pathologies. In addition to Th2 cells, IL-10 is now known to be produced by many cell types including Tr1 cells, CD25+ and CD25− T regulatory cells, Th1 cells, macrophages and dendritic cells as well as B lymphocytes [42]. Induction of IL-10 in response to *T. gondii* infection and particularly in BAL cells has been reported previously [43,44]. Conventional T-bet+forkhead box P3– Th1 lymphocytes raised in spleen and peritoneum after i.p. *T. gondii* infection are also capable of secreting biologically active IL-10. The same cell that secretes IFN-γ to fight infection secretes IL-10 to regulate the immune response against the parasite [43]. Whether these cells, or others, are present and responsible for the diminished allergic airway inflammation observed in our model remains to be explored. Also, in C57BL/6 mice Voisin *et al.* [44] observed a state of hyporesponsiveness in lung during acute *T. gondii* infection. They showed that pulmonary adherent leucocytes from infected mice secreted higher levels of IL-10 compared with noninfected animals. In addition, they described the presence of a population of GR1+ CD11b+ myeloid cells with nitric oxide-dependent regulatory properties. Herein, the source of the increased concentration of IL-10 found in BAL lavage could be those lung-adherent leucocytes described by Voisin *et al.* [44]. However, here, cytokine production was measured at the chronic phase where until now no suppression has been described. It should be taken into account that the mouse strain they used was different from ours. We use BALB/c mice, which are resistant to *T. gondii* infection, while Voisin *et al.* [44] analysed the lung in C57BL/6 mice, a highly susceptible strain.

Finally, this model presents a novel observation. Our data show that the development of allergic airway inflammation in this mouse model can be prevented when OVA sensitization occurs during both stages of *T. gondii* infection. The high levels of IFN-γ induced by the parasite along with the reduction of allergen-specific Th2-associated cytokines and IgG isotypes suggests that the protective effect is related to the highly developed concentrations of Th1 cytokines associated with the *T. gondii* immune response. Nevertheless, the regulatory cytokine IL-10 found in BAL might also be a critical player. These results support epidemiological data showing that *T. gondii* infection correlates with a lesser prevalence of respiratory allergies in humans. It remains to be confirmed whether this diminished lung allergy inflammation also correlates with lower airway hyperresponsiveness observed in asthma. Knowledge of the mechanisms participating in this process will contribute to building up strategies to immunoregulate responses to aeroallergens.

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